C500 Rotation Report

Expression and Eotaxin Binding of ROP^{S55} $CCR3^{E1C-E2C}$

Indiana University

Department of Chemistry

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By:

Jonathan M. Meyers

Introduction:

Chemokines, or chemotatic cytokines, are are a family of glycoproteins that range from around 70 to 90 amino acids in length and have a mass of about 8 to 10 kDa. There are four classes of chemokines that are divided according the order of their two terminal cysteine residues (CC, CXC, CX3C, and C). These small secreted proteins bind to their cognate receptors which are membrane bound proteins. Chemokine receptors are part of a class of proteins named "G-protein coupled receptors" (GPCRs). These transmembrane proteins have seven transmembrane helices and are linked on the interior of the cell to g-proteins. CPCRs bind their cognate ligands on the exterior surface of the cell membrane and transfer the signal to the interior space, thereby signaling a cascade via the G-proteins.

Chemokines attract, activate and regulate tissue infiltration by leukocytes. This is a key component of the inflammatory response. Chemokines are expressed in inflamed tissue, and are ligands for chemokine receptors, which are expressed on the membranes of leukocytes [1]. The inflammatory response includes a change in the expression level of certain chemokines in the leukocytes. Chemokine receptors are also required for the infection of Leukocytes by the human immunodeficiency virus (HIV-1) [4]. These interactions also play a role in the inflammation of the bronchial tubes in an asthmatic episode [8]. The elucidation of the interaction of chemokines and their cognate receptors would be beneficial in the treatment of both of these diseases as wells as providing insight into the mechanism of tissue inflammation.

Background:

Eotaxin , a CC chemokine, is a selective stimulus for eosinophil leukocytes. This chemokine induces the migration of eosinophils in vitro and eosinophil accumulation in vivo [5,7]. Eotaxin is an agonist of the CC chemokine receptor 3 (CCR3) the most abundant chemokine receptor on eosinophils [3]. Binding of Eotaxin to CCR3 has been linked to allergic asthma and allergic rhinitis [7].

The determination of the interaction of Eotaxin with CCR3 has been difficult to elucidate, because CCR3 in an integral membrane protein which makes it hard to isolate and crystallize [10]. To overcome this barrier a soluble protein that mimics the ligand binding elements of GPCRs was designed, prepared, and characterized. Named CROSS (chemokine receptor elements on a soluble scaffold), these proteins consisted of two of the ligand binding elements of CCR3, on the surface of the B1 domain of *Streptococcal* protein G (Fig. 1) [2]. The B1 domain is small thermostable (~6 kD, $\Delta G_{unfolding}$ = 28 kJ/mole at 30^{o}C) [6]. Previous work reported the synthesis of three CROSS proteins named CROSS^{1}-CROSS^{7}, these CROSS proteins only had the N-terminal and E3 segments of CCR3. The most favorable CROSS protein was CROSS^{5}, which incorporated the Mayo hydrophobic core, a hyperstable B1 domain mutant, and a disulfide bond between β-strands β_1 and β_2 . CROSS^{5} was found to have a K_D equilibrium constant of 2.9 ± 0.8 μM [2] for binding to eotaxin.

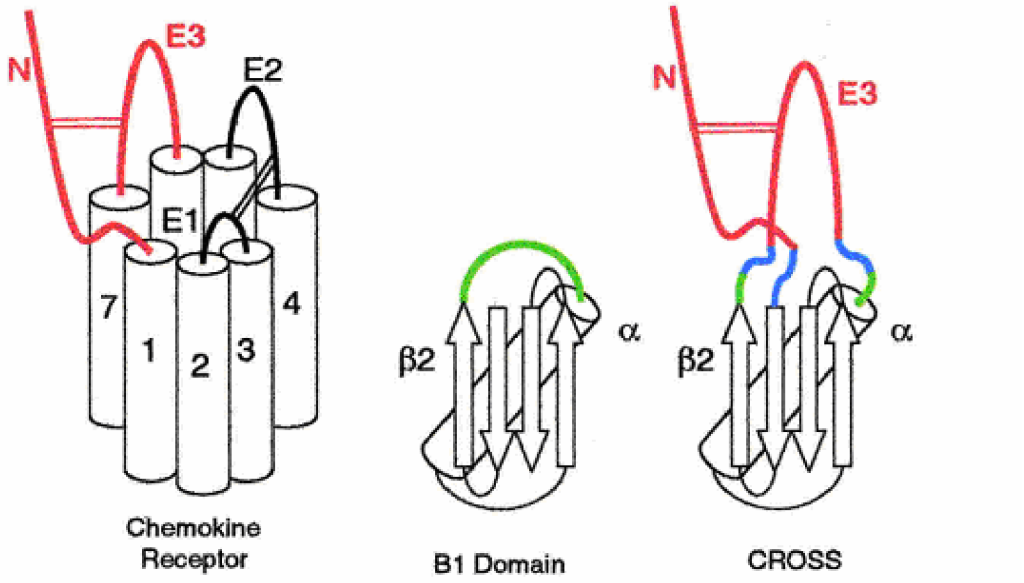

Fig 1

Representation of a Chemokine receptor, the B1 domain used in CROSS, and a CROSS protein with the N-terminal and E3 elements of the Chemokine receptor.

Datta, A., Stone, M.J., Protein Sci. 2003; 12:2482-2491. figure 1.

It has been postulated that the low affinity of CROSS[5] for Eotaxin is a result of the exclusion of the E1 and E2 regions of CCR3 from the CROSS proteins. To determine the effect of the E1 and E2 domains of CCR3 in binding Eotaxin, it was decided that CROSS proteins that contained both the E1 and E2 regions of CCR3 should be synthesized and their binding affinities measured. The scaffold for these proteins is the single-chain variant of ROP developed by Lynn Regan and colleagues. Cecelia Cheng cloned, expressed and purified twelve CROSS proteins with different combinations of the E1 and E2 domains as well as replacing the native Cys in the E1 or E2 domain with a Ser residue. The Goal of this project is to determine whether the soluble protein containing the E1 and E2 elements of CCR3, ROP^{S55} $CCR3^{E1C\text{-}E2C}$ binds to the chemokine Eotaxin, by utilizing isothermal titration calorimetry (ITC), and to determine K_D, stochiometry, and thermodynamic values (ΔH, ΔS, ΔG) for binding.

Results and Discussion:

Preparation and Purification of CROSS

ROP^{S55} CCR3$^{E1C-E2C}$ had previously been cloned, expressed and purified by Cecelia Cheng. The protein was re-lyophilized to remove condensation.

Preparation and Purification of Eotaxin

Eotaxin of unknown purity was available from Michael Goodman. The protein solution was condensed and purified by HPLC using a C_4 column, in acetonitrile buffers. SDS page electrophoresis was used to determine the purity of Eotaxin fractions.

ITC Experiment

Eotaxin and ROP^{S55} CCR3$^{E1C-E2C}$ were dialyzed against 50mM MOPS buffer at pH 7. After dialysis the concentration of each protein was determined by using their respective OD_{280}. An aliquot of Eotaxin was then diluted to give 300µL of a 500 µM solution. An aliquot ROP^{S55} CCR3$^{E1C-E2C}$ was diluted to give 2.0 mL of a 10 µM solution. ITC experiments were to be carried out on a Microcal VP-ITC instrument. All solutions were thouroghly degassed before ITC was carried out. Eotaxin was injected into the calorimeter well and ROP^{S55} CCR3$^{E1C-E2C}$ was to be drawn into the syringe, but when the ROP solution was drawn into a Hamiliton syringe it was noticed that the protein was aggregating and precipitating out of solution. Several attempts to soluablizie the protein failed. The stock of ROP^{S55} CCR3$^{E1C-E2C}$ had been depleted, therefore ITC experiments were postponed until more ROP^{S55} CCR3$^{E1C-E2C}$ could be expressed and purified.

Expression and Purification of ROP^{S55} CCR3$^{E1C-E2C}$

Expression and purification was performed following the protocol set for by Ceclia Cheng for expression of ROP^{S55} CCR3$^{E1C-E2C}$. Expression was performed using BL21 cells, and a four liter growth was performed. Upon purification none of the desired protein was seen. One possible explanation is that it crashed off the column immedialty upon loading the column and was lost.

Experimental:

Preperation and Puification of ROP^{S55} CCR3$^{E1C-E2C}$

ROP^{S55} CCR3$^{E1C-E2C}$ was cloned, expressed and purified by Ceclia Cheng. The lyophilized was dissolved in 1.0 mL of H_2O and the OD_{280} used to determine the concentration.

Preperation and Purification of Eotaxin

Eotaxin was cloned, expressed and purified by Michael Goodman. Seventeen milliliters of Eotaxin solution was concentrated to approximately 5 mL. The solution was purified in four aliquots using a C_4 column on a HPLC apparatus. The first aliquot was 2.0 mL, but overloaded the detector. SDS page gel electrophoresis was used to determine which fractions contained the Eotaxin. Three subsequent aliquots of 1 mL apiece were purified on the C_4 column. SDS gel electorphoresis was used to confirm purification. The fractions from each purification that contained Eotaxin was pooled and then concentrated to 2.0 mL and the concetration determined using it OD_{280}.

ITC

The Eotaxin and ROP^{S55} $CCR3^{E1C-E2C}$ solutions were then dialyzed for 24 hours in 2.0L of 50mM MOPS buffer at pH 7, with 0.02% NaN_3 added to prevent bacteria growth. After dialysis the concentration of both solutions was again determined using their OD_{280}. An aliquot of Eotaxin was diluted with the dialysis buffer to give 300 µL of 500 µM solution. An aliquot of ROP^{S55} $CCR3^{E1C-E2C}$ was diluted with dialysis buffer to give 2.0 mL of 10 µM solution. Both solutions were degassed by stirring for two hours under vacuum. The Eotaxin solution was transferred to the ITC reaction well, and the ROP solution was to be titrated in. Aggregation and precipitation of the ROP prevented the completion of the ITC experiment.

Expression and Purification of ROP^{S55} $CCR3^{E1C-E2C}$

Fifty millilites of LB was inoculated with one colony of Rop, and 50 µL of Kan. was added. The starter culture was then transferred to 500 mL flasks and 500 uL of Kan. added. When the OD_{280} reached 0.600 the cells were induced with 5 mL of 100 mM IPTG. After 6.5 hours the cells were spun down at 8 K for 10 min. Cells were resuspended in 20 mL ROP lysis buffer (Table 1). One hundred and twenty mirco liters of 50 mM lysomzyme and PMSF was added and then incubated on ice for 10 min. The cells were sonicated five times with a large tip, 30 sec/1 min rest cycles at level 4.5. The cells were then spun down at 17 K for 20 min. The pellets were resuspended in resuspension solution (Table 1). The resuspended pellets were incubated for one hour on ice, then spun down a second time at 17 K for 20 min. After centrifugation the pellets were resuspended in resuspension solution. Supernatant was dialyzed in 4 L of refolding buffer (Table 2) for 12 hours at room temperature. The solution was then transferred and dialyzed in refolding

buffer B (Table 1) for 12 hours at room temperature. Then the solution was transferred and dialyzed into the final buffer (Table 1) for 12 hours at room temperature. After final dialysis the solution was concentrated to 100 mL. Final solution was purified on a FPLC Q column. No product was seen.

Solution	
ROP Lysis Buffer pH 7.4	50 mM Tris-HCl, 2 mM EDTA, 10mM $CaCl_2$, 0.5 mM DTT
Resuspension Solution pH 4.0	10 mM Tris-HCl, 8 M Urea, 100 mM NaH_2PO_4
Refolding Buffer A pH 8	50 mM Tris-HCl, 50 mM glycine, 500 mM NaCl, 3 M Urea, 0.4 mM oxidized glutathione, 4.0 mM reduced glutathione
Refolding Buffer B pH 8	50 mM Tris-HCl, 50 mM glycine, 500 mM NaCl, 1 M Urea, 0.5 mM oxidized glutathione, 2.4 mM reduced glutathione
Final Buffer pH 8	20 mM Tris-HCl, 150 mM NaCl

Table 1: Solutions used in Expression of ROP^{S55} $CCR3^{E1C-E2C}$.

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